The family of Peps and their precursors in Arabidopsis: differential expression and localization but similar induction of pattern-triggered immune responses

Bartels, S ; Lori, M ; Mbengue, M ; van Verk, M ; Klauser, D ; Hander, T ; Boni, R ; Robatzek, S ; Boller, T

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RESEARCH PAPER

The family of Peps and their precursors in *Arabidopsis*: differential expression and localization but similar induction of pattern-triggered immune responses

Sebastian Bartels[1,*], Martina Lori[1], Malick Mbengue[2], Marcel van Verk[3], Dominik Klauser[1], Tim Hander[1], Rainer Böni[1], Silke Robatzek[2] and Thomas Boller[1]

[1] Zürich-Basel Plant Science Center, University of Basel, Department of Environmental Sciences, Botany, Hebelstrasse 1, CH-4056 Basel, Switzerland
[2] The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, UK
[3] Plant–Microbe Interactions, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

* To whom correspondence should be addressed. E-mail: sebastian.bartels@unibas.ch

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Abstract

In *Arabidopsis thaliana*, the endogenous danger peptides, *At*Peps, have been associated with plant defences reminiscent of those induced in pattern-triggered immunity. *At*Peps are perceived by two homologous receptor kinases, PEPR1 and PEPR2, and are encoded in the C termini of the PROPEP precursors. Here, we report that, contrary to the seemingly redundant *At*Peps, the PROPEPs fall at least into two distinct groups. As revealed by promoter–β-glucuronidase studies, expression patterns of *PROPEP1–3*, *-5*, and *-8* partially overlapped and correlated with those of the *PEPR1* and *-2* receptors, whereas those of *PROPEP4* and *-7* did not share any similarities with the former. Moreover, bi-clustering analysis indicated an association of *PROPEP1*, *-2*, and *-3* with plant defence, whereas *PROPEP5* expression was related to patterns of plant reproduction. In addition, at the protein level, PROPEPs appeared to be distinct. PROPEP3::YFP (fused to yellow fluorescent protein) was present in the cytosol, but, in contrast to previous predictions, PROPEP1::YFP and PROPEP6::YFP localized to the tonoplast. Together with the expression patterns, this could point to potentially non-redundant roles among the members of the PROPEP family. By contrast, their derived *At*Peps, including the newly reported AtPep8, when applied exogenously, provoked activation of defence-related responses in a similar manner, suggesting a high level of functional redundancy between the *At*Peps. Taken together, our findings reveal an apparent antagonism between *At*Pep redundancy and PROPEP variability, and indicate new roles for PROPEPs besides plant immunity.

Key words: *Arabidopsis*, *At*Pep, DAMP, danger peptide, endogenous elicitor, PROPEP, PTI.

Introduction

Danger- or damage-associated molecular patterns (DAMPs) are diverse molecules, which trigger the immune system upon perception (Scaffidi *et al.*, 2002; Seong and Matzinger, 2004; Ahrens *et al.*, 2012). Unlike microbe/pathogen-associated molecular patterns (MAMPs/PAMPs), which originate from microorganisms, DAMPs are endogenous molecules of the host (Boller and Felix, 2009). In animals, DAMPs can be produced in the context of damage as degradation products of proteins, DNA, or the cytoskeleton (Ahrens *et al.*, 2012; Pisetsky, 2012), or they are signals associated with danger

Abbreviations: DAMP, damage-associated molecular pattern; GUS, β-glucuronidase; MAMP, microbe-associated molecular pattern; MAPK, mitogen-activated protein kinase; OG, oligogalacturonides; PRR, pattern recognition receptor; PS, prosystemin; PTI, pattern-triggered immunity; YFP, yellow fluorescent protein.

and thus are actively released (Wang *et al.*, 1999). The latter DAMPs are reminiscent of cytokines such as interleukins, which are processed and released upon an imminent threat, for example the detection of MAMPs (van de Veerdonk *et al.*, 2011). In plants, much less is known about potential DAMPs or cytokine-like proteins. Paradigms of plant DAMPs are cell-wall degradation products such as oligogalacturonides (OGs), which trigger pattern-triggered immunity (PTI) upon detection (Rasul *et al.*, 2012). They are released by the activity of microbe-secreted cell-wall-degrading enzymes and perceived by transmembrane pattern recognition receptors (PRRs) (D'Ovidio *et al.*, 2004). Besides these prototype DAMPs, endogenous peptides have been identified that trigger a PTI-like response as well.

The systemins from the Solanoideae, a subfamily of the Solanaceae that includes tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), and potato (*Solanum tuberosum*), were the first plant hormones identified to induce the accumulation of proteinase inhibitors, a typical anti-herbivore response, and later connected to the regulation of diverse defence responses (Pearce *et al.*, 1991; McGurl *et al.*, 1992; Ryan and Pearce, 2003). Tomato systemin is an 18 aa peptide processed from a 200 aa precursor protein called prosystemin (PS). Despite many years of systemin research, the systemin receptor is still a matter of debate (Holton *et al.*, 2008; Lanfermeijer *et al.*, 2008; Malinowski *et al.*, 2009). Recently, the *PS* gene from tomato was shown to be expressed mainly in floral tissues, especially pistils, anthers, and sepals, and only at lower levels in leaves. Treatment of leaves with methyl jasmonate led to a strong induction of *PS* expression (Avilés-Arnaut and Délano-Frier, 2012). Similar to the expression patterns, PS protein was constitutively found in floral organs including sepals, petals, and anthers, as well as in the vascular phloem parenchyma cells of leaves and stems, where it localizes to the cytosol and the nucleus (Narváez-Vásquez and Ryan, 2004).

DAMP- or cytokine-like peptides have also been found in *Arabidopsis thaliana*. Two of these 23 aa peptides, AtPep1 and AtPep5, have been purified from *Arabidopsis* leaf protein extracts (Huffaker *et al.*, 2006; Yamaguchi and Huffaker, 2011). They belong to a small family of seven homologous peptides, which comprise the C-terminal part of seven small precursor proteins called PROPEPs (Huffaker and Ryan, 2007). It is believed that the PROPEPs are cleaved to release the *At*Peps, which in turn are perceived by the two homologous receptor-like kinases PEPR1 and PEPR2 (Yamaguchi *et al.*, 2010; Krol *et al.*, 2010). Upon detection, the PEPRs trigger a set of responses reminiscent of PTI including induced resistance against subsequent infections with virulent *Pseudomonas syringae* bacteria (Huffaker and Ryan, 2007; Krol *et al.*, 2010; Yamaguchi *et al.*, 2010). In addition to the classical PTI-associated responses, recent data show that treatment with AtPep3 led to an increase in cytosolic cGMP, suggesting that *At*Peps activate cGMP-dependent signalling pathways (Qi *et al.*, 2010).

To date, little is known about the expression, localization, and function of the PROPEPs. The expression of a number of *PROPEP*s is induced upon treatment of *Arabidopsis* leaves with methyl jasmonate and methyl salicylate, as well as MAMPs and *At*Peps (Huffaker and Ryan, 2007). At the cellular level, PROPEPs are thought to reside in the cytosol and be exported to the extracellular space via an unconventional secretion system, as the PROPEPs carry no known secretion or subcellular localization signals (Yamaguchi and Huffaker, 2011; Ding *et al.*, 2012).

In this study, we focused on the PROPEPs, including an additional eighth member of the PROPEP family in *Arabidopsis*, reported here for the first time. Our data demonstrated that all eight *At*Peps elicited PTI-type responses in a similar manner and depended on the PEPR1/2 receptor pair, revealing great functional redundancy. By contrast, bi-clustering analysis, promoter–β-glucuronidase (GUS) expression and PROPEP::YFP (fused to yellow fluorescent protein) localization studies identified significant tissue-specific differences and subcellular patterns that highlight potentially non-redundant properties of the precursors. Furthermore, our data led to the idea that some PROPEPs might play a role in plant development and reproduction, in addition to their described function in plant immunity.

Materials and methods

Plant material

Mature *Arabidopsis* plants were grown in individual pots at 21 °C and an 10 h photoperiod for 4–5 weeks. For induction of flowering, plants were moved to a 16 h photoperiod. For preparation of sterile seedlings, *A. thaliana* seeds were surface sterilized with 70% ethanol and plated on half-strength Murashige and Skoog (MS) medium supplemented with 1% sucrose and 0.5% Phytagel (Sigma-Aldrich), stratified for at least 2 d at 4 °C, and then germinated at 21 °C in continuous light (MLR-350; Sanyo). The T-DNA insertion lines SALK_059281 (*pepr1*) and SALK_098161 (*pepr2*) were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK) and are in the Col-0 accession background.

Generation of transgenic Arabidopsis *lines*

The PROPEP and PEPR putative promoter sequences were amplified by PCR from genomic Col-0 DNA with specific primers (see Supplementary Table S4 at *JXB* online for primers and promoter sequences). The obtained sequences were introduced into the binary destination vector pBGWFS7 (Karimi *et al.*, 2002) using Gateway-based cloning. *PROPEP*s were cloned from Col-0 cDNA using gene-specific primers (Supplementary Table S4). Introducing PROPEP sequences into the binary destination vector pEarley101 by Gateway-based recombination led to the in-frame fusion of YFP to their C-terminal ends (Earley *et al.*, 2006). *Arabidopsis* plants were transformed by *Agrobacterium tumefaciens* using the floral dip method (Clough and Bent, 1998).

Peptides

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA), AtPep1 (ATKVKAKQRGKEKVSSGRPGQHN), AtPep2 (DNKAKSKKRDKEKPSSGRPGQTNSVPNAAIQVYKED), AtPep3 (EIKARGKNKTKPTPSSGKGGKHN), AtPep4 (GLPGKKNVLKKSRESSGKPGGTNKKPF), AtPep5 (SLNVMRKGIRKQPVSSGKRGGVNDYDM), AtPep6 (ITAVLRRRPRPPPYSSGRPGQNN), AtPep7 (VSGNVAARKGKQQTSSGKGGGTN), AtPep8 (GGVIVKSKKAARELPSSGKPGRRN) obtained from EZBiolabs were dissolved in a solution containing 1 mg ml^{-1} of bovine serum albumin

and 0.1 M NaCl to get peptide stocks of 100 µM. Further dilutions were done with water.

Microarray and data analysis

Bi-clustering and co-expression analysis was performed as described by van Verk *et al.* (2011), with the following minor modifications: for bi-clustering, the Euclidean distance measure was used. To obtain separate clusters containing the *PROPEP*s, the first cluster within the dendogram containing less than 500 genes was selected. For gene annotations into biological categories, the AmiGO Term Enrichment software was employed (Carbon *et al.*, 2009). For categorization of enriched gene ontology (GO) terms, the CateGOrizer tool (Hu *et al.*, 2008) using Plant GO-Slim terms was used, applying the consolidated single occurrences count option. Supplementary Table S2 at *JXB* online provides a list of the Affymetrix 25K microarrays from NASCArrays and AtGenExpress (downloaded from ftp://ftp.arabidopsis.org/).

GUS staining

Plant tissue was fixed in ice-cold 90% acetone for 20 min, washed with water, and then placed in GUS staining buffer [1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (Gold BioTechnology, St Louis, Missouri, USA), 100 mM sodium phosphate (pH 7.5), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, and 0.1% (v/v) Triton X-100] at 37 °C for 2 h (seedlings) or 24 h (adult leaves). Plant tissue was cleared with 70% (v/v) ethanol and photographed using an Olympus SZX12 binocular microscope in combination with an Olympus DP72 camera and the CellSens imaging software (Olympus America, Pennsylvania, USA).

Fluorescence microscopy

Seven-d-old seedlings expressing the PROPEP::YFP and Pep1::YFP fusions were stained for 5 min in an aqueous solution containing FM4-64 (SynaptoRed; Sigma-Aldrich) diluted at 5 µg ml^{-1} and washed for 5 min in water prior to imaging using an SP5 Leica confocal microscope. YFP (500–560 nm) and FM4-64 (620–650 nm) fluorescence was recorded simultaneously after excitation at 488 nm using a ×63 water-immersion objective. Plasmolysis was achieved by mounting roots in 500 mM NaCl solution for 2 min prior to imaging.

Measurement of ethylene production

For measurement of ethylene accumulation, five seedlings (5 d after germination) were harvested into a 6 ml glass vial containing 0.1 ml of ddH_2O, placed back into the growth chamber, and left overnight (~16 h). Peptides were added to 1 µM final concentration and the vials were closed and made air-tight with rubber septa. After 5 h of incubation on a shaker (100 rpm) at room temperature, ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu).

Mitogen-activated protein kinase (MAPK) phosphorylation

Ten seedlings (10 d after germination) were placed into 0.5 ml of sterile water and left floating overnight (16 h). Peptides were added to a final concentration of 1 µM. After 15 min, seedlings were shock frozen and ground to a fine powder before the addition of 80 µl of extraction buffer [0.35 M Tris/HCl (pH 6.8), 30% (v/v) glycerol, 10% SDS, 0.6 M dithiothreitol, 0.012% (w/v) bromphenol blue]. After boiling for 5 min, 10 µl of the total cellular protein extract was separated by 12% SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride membrane according to the manufacturer's instructions (Millipore). We used primary monoclonal antibodies against phospho-p44/42 MAP kinase (Cell Signaling Technologies) and actin (Sigma-Aldrich), with alkaline phosphatase-conjugated anti-rabbit and anti-mouse immunoglobulins (Sigma-Aldrich) as secondary antibodies, as required. Signal detection was performed using CDPstar (Roche).

Growth inhibition assays

At 5 d after germination, sterile seedlings were transferred to liquid MS medium supplied with the peptides at 1 µM final concentration (one seedling per 500 µl of medium in 24-well plates). The effect of treatment with different peptides on seedling growth was analysed after 10 d by determining fresh weight.

Results

AT5G09976 is a novel member of the Arabidopsis *PROPEP family*

In order to gain insights into the sequence homology of PROPEPs compared with other precursors of plant signalling peptides, we searched the *Arabidopsis* genome and identified AT5G09976 as a new member of the PROPEP family. It clustered with the other seven PROPEPs, despite an overall low sequence homology, and its C terminus contained the conserved *At*Pep motif SSG-x_2-G-x_2-N (Fig. 1A). According to sequence similarity, PROPEP4 was the closest homologue of AT5G09976. Moreover, addition of a synthetic peptide based on the last 23 aa of AT5G09976 (Fig. 1A, highlighted with a red bar) triggered similar responses in *Arabidopsis* plants to the other *At*Peps (see below). Thus, we designated AT5G09976 as *PROPEP8*. Further searches for non-annotated sequences with similarity to the PROPEPs did not reveal any further PROPEP in *Arabidopsis*.

Bi-clustering expression analysis indicates distinct roles for individual PROPEPs

PROPEPs are thought to assist via the release of *At*Peps in biotic stress resistance, but their individual roles have not been investigated in detail (Huffaker and Ryan, 2007; Boller and Felix, 2009). Whereas *At*Peps are assumed to act rather redundantly, little is known about the spatial and temporal expression of PROPEPs. It has been shown that PROPEPs respond with slight differences to treatments with methyl jasmonate, methyl salicylate, and *At*Peps (Huffaker and Ryan, 2007). In order to get a better idea about potential redundance as well as specific expression patterns of the PROPEPs in the context of biotic stress resistance, we performed a bi-clustering analysis focusing on 278 biotic stress-related microarrays that were downloaded from the TAIR website (ftp://ftp.arabidopsis.org/Microarrays/analyzed_data). Thus, the 22 810 probes (representing genes) present on the Affymetrix 25K arrays used were grouped based on their expression patterns over the various biotic stress treatments. Genes with similar expression patterns grouped more closely together, as indicated by the dendrogram, and became part of a subgroup (subclusters). Enrichment of GO terms within one subcluster could be used to get indications about the function of the genes in this subcluster. Moreover, the relative distance of genes within the main cluster showed the diversity of regulation of these genes.

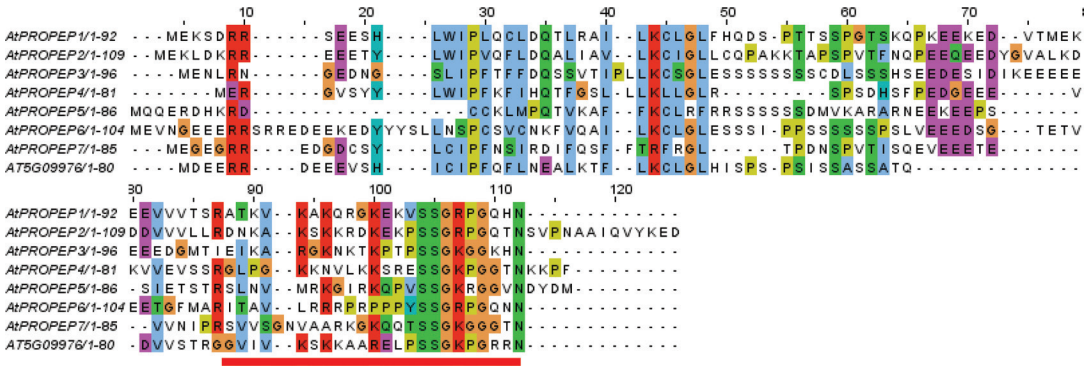

Fig. 1. Alignment of the eight *Arabidopsis* PROPEPs. ClustalW alignment of the amino acid sequences of all identified *Arabidopsis* PROPEPs including AT05G09976. Colouring is based in the Clustal colour scheme. (This figure is available in colour at *JXB* online.)

PROPEP-containing clusters were selected by setting an individual cut-off within the dendrogram for each *PROPEP* gene to yield a cluster with <500 genes (Fig. 2). *PROPEP7* and *PROPEP8* are not spotted on the arrays used, and therefore no bi-cluster analysis could be performed for these precursors. Of the family members *PROPEP1–6*, only *PROPEP2* and *-3* clustered together, indicating that most of the *PROPEP*s are expressed in different ways upon treatment with various biotic stimuli. To get an indication of which processes the individual *PROPEP*s are involved in, a GO term enrichment analysis was performed on the obtained clusters, the top five terms of which are shown in Fig. 2. Most enriched GO terms within the top five of each cluster represented a relatively broad description of a process. To also provide data on the more specific processes that are underlying the expression of these clusters, a full overview of all major and minor enriched GO terms for each *PROPEP* is provided in Supplementary Table S1 at *JXB* online. As most *PROPEP*s appeared to be involved in very different processes besides biotic stress resistance, a co-expression analysis followed by a GO term analysis on a set of abiotic or development-related microarrays was also performed (Supplementary Table S2 at *JXB* online). These results further supported the idea that *PROPEP* transcription seems to be regulated individually and does not follow a general pattern valid for all *PROPEP*s.

The *PROPEP* that had the most similar global expression pattern compared with *PROPEP2* and *-3* was *PROPEP1*, but, besides the shared enriched defence-associated GO terms (Fig. 2), they also had some characteristically different enriched GO terms. The *PROPEP1* cluster revealed an additional enrichment of GO terms related to abiotic stress, hypoxia, and abscisic acid signalling, whereas the *PROPEP2* and *-3* cluster was also associated with salicylic acid signalling, (programmed) cell death, and (transmembrane) ion transport (Supplementary Tables S1 and S2). Distinct from these more defence-associated *PROPEP*s was the cluster of *PROPEP5* that was enriched for processes related to reproduction and shared the enrichment for gibberellin/terpenoid biosynthesis and lipid signalling with *PROPEP6*. The most directly noticeable different *PROPEP* in Fig. 2 was *PROPEP4*, whose expression was induced in conditions where all the other *PROPEP*s were repressed and vice versa. As the cluster of *PROPEP4* contained only 25 genes, it was too small to result in any enriched GO terms. To circumvent this, the genes that were co-expressed with *PROPEP4* given a Pearson correlation coefficient cut-off of >0.60 were used to perform a GO term enrichment analysis. This resulted in an enrichment of organismal development-, developmental processes-, and chromosome/chromatin organization-associated GO terms.

To further diversify the view on *PROPEP* regulation, we also analysed the type of treatments and/or conditions that had the strongest influence on the expression of each *PROPEP*; a full overview of treatments, conditions, and their influence on expression is given in Supplementary Table S3 at *JXB* online. Here we found, in agreement with the bi-clustering analysis, *PROPEP1* to be highly induced by abiotic stress treatments like salt, drought, and osmotic stress, whereas for example, *PROPEP5* transcription was highest in certain developmental stages of seeds and flowers.

Overall, our analysis indicated that, in contrast to *At*Peps, the transcriptional regulation of *PROPEP*s is most likely non-redundant. Moreover, based on these findings, we suggest that individual PROPEPs could play a role in very distinct functions in *Arabidopsis*, as they appear to be associated not only with defence but also with processes ranging from abiotic stress resistance to development and reproduction.

Analysis of PROPEP *promoters reveals diverse spatial and temporal expression patterns*

To investigate further the potential difference in *PROPEP* expression at the tissue level, we generated transgenic *Arabidopsis* lines containing the putative promoter sequences of the *PROPEP* genes fused to GUS. As shown in Fig. 3, the promoters of *PROPEP1*, *-2*, and *-3* exhibited similar expression patterns. These promoters conferred expression mainly in the root excluding the root tip. In adult leaves, even after 24 h of staining, nearly no blue precipitate was visible indicating, very low activity of these promoters without stimuli. In contrast, wounding of leaves using forceps led to a clear induction of these *PROPEP* promoters, which was restricted to the vasculature (Fig. 3, yellow arrows). Besides the great overlap between the

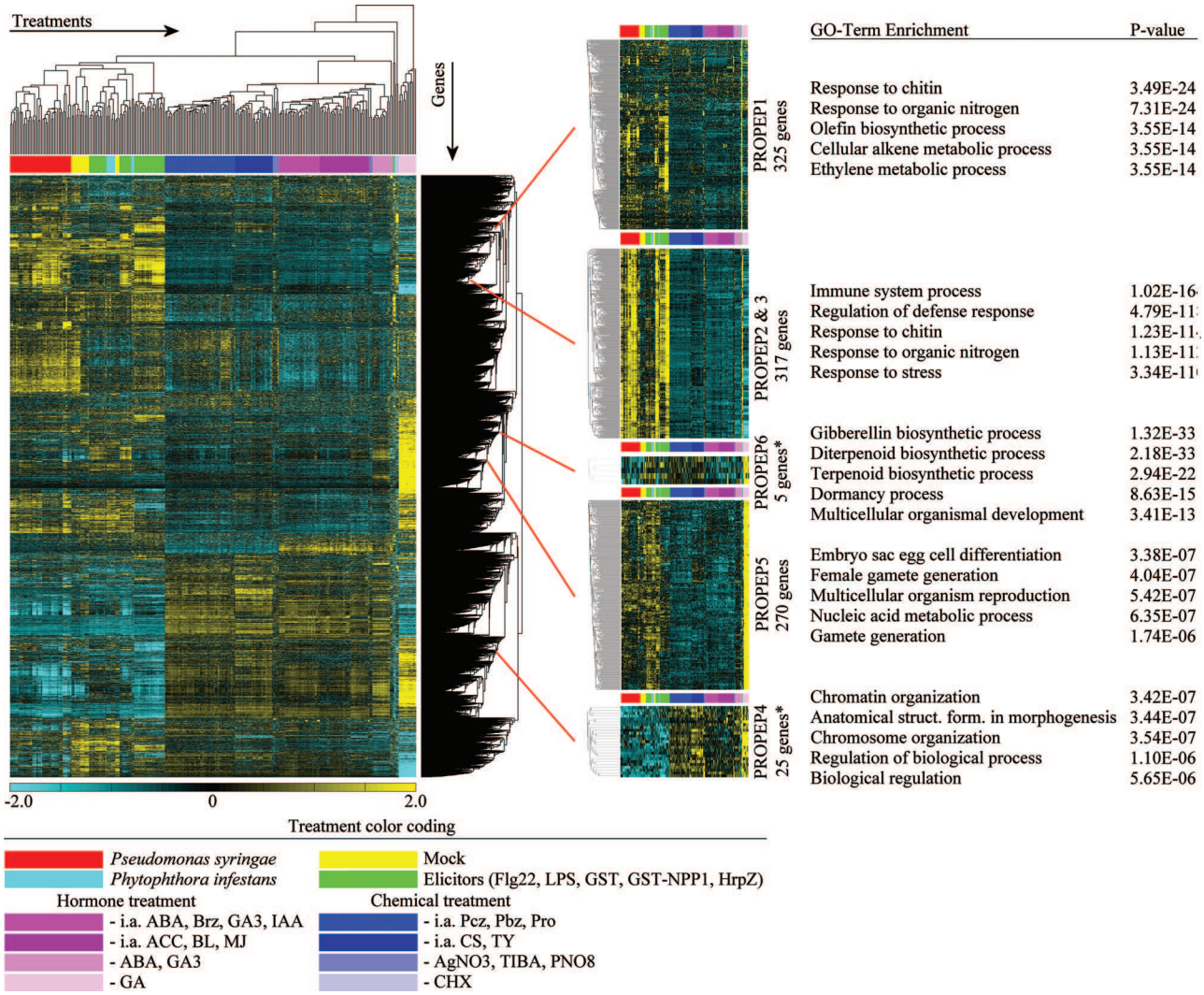

Fig. 2. Bi-clustering analysis of *PROPEP*s based on expression profiles of biotic stress treatments. The similarity in expression pattern of 22 810 different probes (representing genes) was assessed by performing a bi-clustering analysis of the $\log_2$-transformed expression values from 278 biotic stress-related microarrays (upregulated genes are represented in yellow, whereas downregulated genes are coloured blue). The different types of treatments within this bi-clustering analysis are colour coded above the clusters, with their details at the bottom of the figure. For multiple treatments, typical examples are given, as each individual treatment could not be colour coded clearly. A full list of all treatments, including the dendrogram and the same colour coding, is given in Supplementary Table S2. Genes that cluster relatively closely are expressed similarly under various biotic stresses and vice versa. Only *PROPEP2* and *-3* clustered very closely together, suggesting that only these two *PROPEP*s are involved in similar processes under biotic stress. To obtain an indication of which processes each *PROPEP* is involved in, GO term enrichment was performed on each subcluster containing a *PROPEP* (represented as separate clusters). The top five enriched GO terms of the subcluster, indicating the related processes, is shown to the right of each subcluster. Asterisks denote subclusters that showed no enriched GO terms; therefore, co-expressed genes with the *PROPEP* having a Pearson correlation coefficient of >0.6 were used for GO term enrichment analysis.

expression patterns of the promoters of *PROPEP1*, *-2*, and *-3*, the latter also produced GUS staining in the anthers of flowers.

The promoters of *PROPEP5* and *-8* were also active in the root but restricted to the vascular tissue, reminiscent of the promoter of *PEPR2* (see below). They shared with the promoters of *PROPEP1–3* the wound inducibility in the central vasculature of adult leaves. However, whereas the promoter of *PROPEP5* showed strong activity in the leaf veins, the promoter of *PROPEP8* did not produce any GUS staining in untreated leaves. In addition, they produced distinct staining in adult flowers. The promoter of *PROPEP5* was highly active in the filaments of flowers (Fig. 3, white arrow), whereas that of *PROPEP8* was active in all flower tissues except for the petals. Thus, the promoters of *PROPEP5* and *-8* partially shared their expression patterns with those of the promoters of *PROPEP1–3* but also showed differences from them and among each other.

Intriguingly, the activity of the promoters of *PROPEP4* and *-7* were restricted to the tips of primary and lateral roots (Fig. 3, red arrows), whereas neither the other *PROPEP* promoters nor the promoters of *PEPR1* and *-2* (see below)

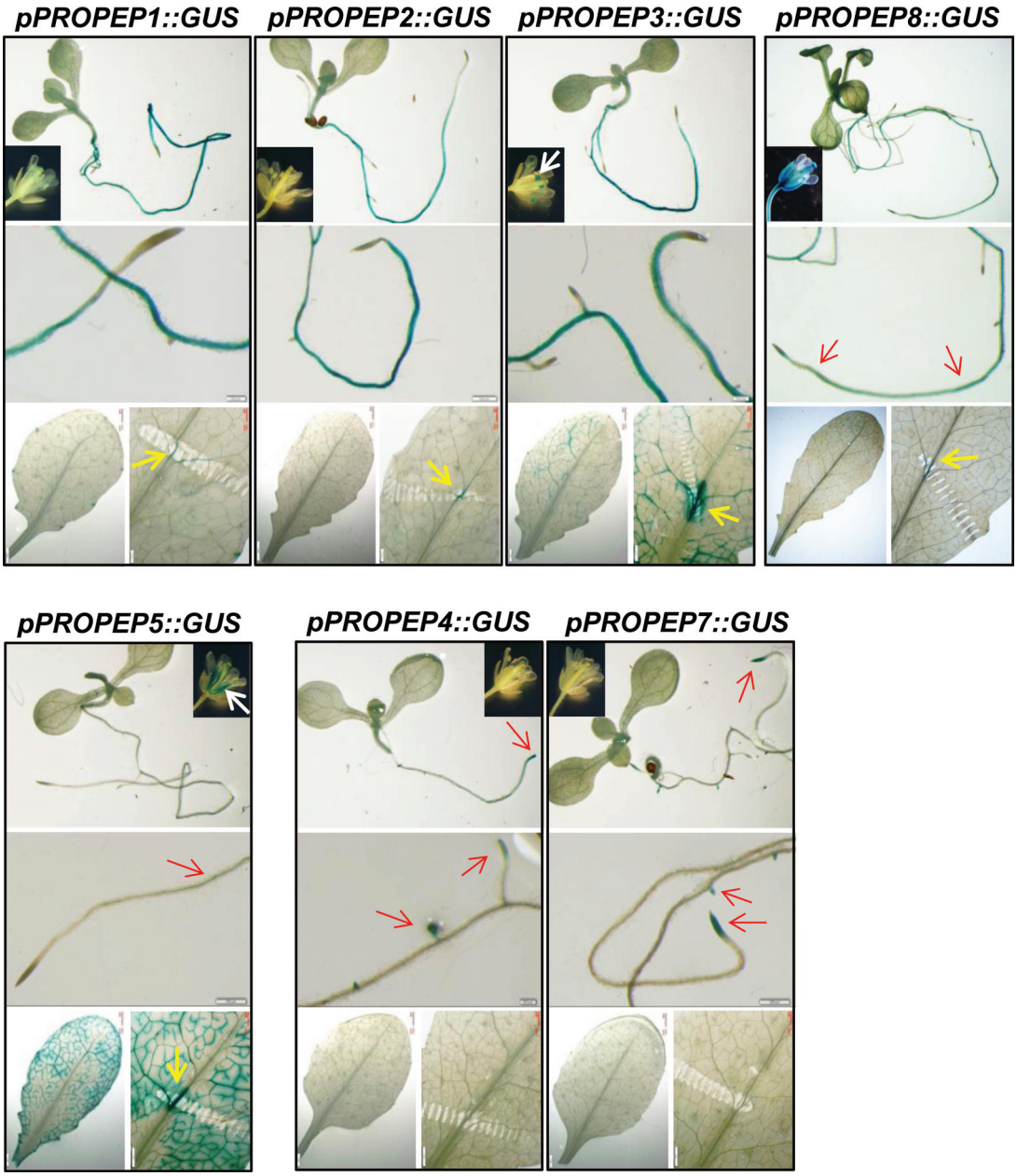

Fig. 3. Spatial and temporal expression patterns of *PROPEP* promoter–GUS lines. Fusion of putative promoter sequences of indicated *PROPEP*s to a GUS reporter revealed distinct expression patterns. Pictures show staining (2 h) of untreated 10-d-old seedlings grown in sterile conditions on MS plates and adult leaves, and flowers of soil-grown plants (24 h staining). Wounding of adult leaves was done with forceps and the plants were incubated for 2 h before staining. Red arrows indicate expression in root tips of the primary and lateral roots. Yellow arrows indicate GUS staining in the vasculature after wounding. White arrows highlight flowers with GUS expression. Three independent transgenic lines were analysed for each construct, showing similar results.

conferred any obvious GUS expression. Moreover, expression of the promoters of *PROPEP4* and *-7* was not detected in flowers and was not induced by wounding.

Taken together, the promoter-mediated expression patterns of the *PROPEP*s fell clearly into two distinct groups. Group 1, which comprised the promoters of *PROPEP1*, *-2*, *-3*, *-5*, and *-8*, showed expression in the roots and slightly in the leaf vasculature. They were inducible by wounding. Group 2, containing the promoters of *PROPEP4* and *-7*, was not inducible by wounding and the basal expression was restricted to the root tips.

PROPEP::YFP fusions identify localization to distinct subcellular compartments

Next we generated transgenic *Arabidopsis* plants constitutively expressing PROPEP::YFP fusion proteins to assess their subcellular localization. It has been hypothesized that all PROPEPs localize to the cytoplasm based on the predicted function and the lack of an identifiable localization signal (Huffaker *et al.*, 2006). In line with this hypothesis, PROPEP3::YFP localized to the cytoplasm (Fig. 4A). However, our findings with PROPEP1::YFP and

PROPEP6::YFP were rather surprising and showed that these precursor proteins were associated with the tonoplast. To clearly distinguish the tonoplast from the plasma membrane, we performed a brief FM4-64 staining (Fig. 4A, red), which is often used to image the plasma membrane. The overlay confirmed that the YFP fluorescence and that emitted from FM4-64 did not overlap.

To exclude the possibility that subcellular localization was dependent on the cell type, we imaged epidermal cells of cotelydons as well as root epidermal cells and observed that

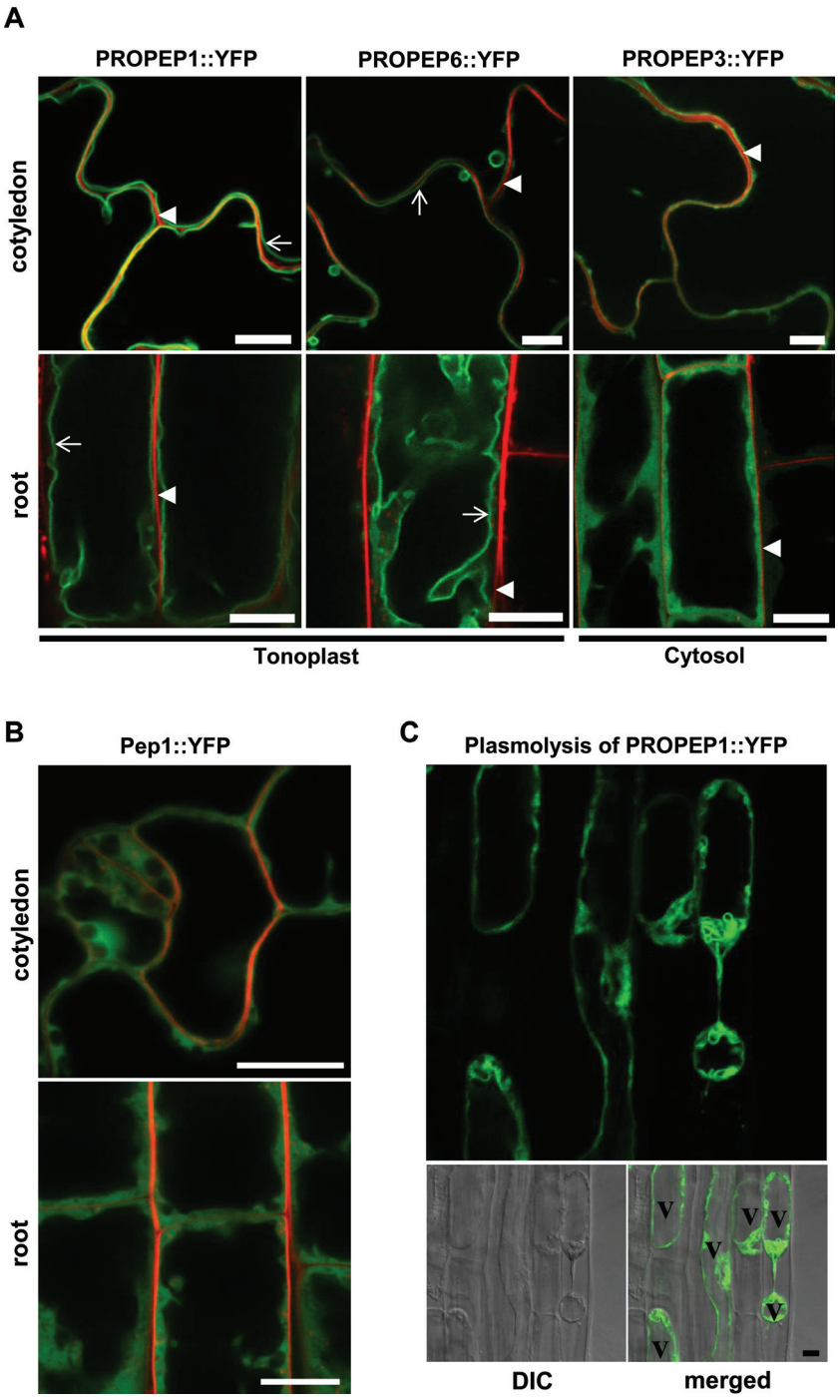

Fig. 4. Subcellular localization of PROPEP::YFP fusion proteins. (A, B) Confocal micrographs of *Arabidopsis* transgenic lines, expressing PROPEP::YFP (A) and Pep1::YFP (B) fusion proteins as indicated under the control of the cauliflower mosaic virus 35S promoter showing single optical sections of cotyledon epidermal cells (top panel) or root epidermal cells (bottom panel). Co-staining with FM4-64 (red channel) highlights the plasma membrane (arrowheads). PROPEP1 and PROPEP6::YFP fusions localize to the tonoplast in both tissues (right panels - arrows) while PROPEP3::YFP (left panel) and Pep1::YFP (B) fusion protein localized to the cytosol in both tissues. Similar results were obtained in two independent transgenic lines for each construct. Bars, 10 μm. (C) Plasmolysis of root cells after 2 min of 500 mM NaCl treatment. DIC, differential interference contrast.

the localization patterns were the same. In contrast, a fusion protein of just the C-terminal part of PROPEP1, which represents AtPep1, with YFP produced a cytosolic localization, indicating that the association of PROPEP1 with the tonoplast seemed to depend on the N-terminal part of PROPEP1 and was not due to binding of AtPep1 to an as yet unidentified tonoplast-localized protein (Fig. 4B).

In order to test further the association of PROPEP1::YFP with the tonoplast, we performed plasmolysis triggered by a brief treatment with 500 mM NaCl. As shown in Fig. 4C, the YFP fluorescence remained at the tonoplast of the shrunken vacuoles.

These findings demonstrated that members of the PROPEP family are present at two different subcellular compartments, the cytosol and the tonoplast. This might indicate non-redundant functions between the PROPEPs at the protein level or an as yet not understood level of complexity of their involvement in cellular immunity. Moreover, it provides evidence for a potential role of PROPEP1 and -6 associated with the vacuole.

The promoters of PEPR1 *and -2 confer overlapping patterns of expression, which resemble those of some but not all* PROPEP *promoters*

*At*Peps are known to be detected by two homologous receptors, PEPR1 and PEPR2 (Krol *et al.*, 2010; Yamaguchi *et al.*, 2010). To investigate the potential overlap of the expression patterns between the two PEPRs and the PROPEPs, we generated transgenic *Arabidopsis* lines containing the putative promoter sequences of the *PEPR* promoters fused to GUS. As shown in Fig. 5, both promoters conferred expression in the vascular tissue of roots and leaves. No *PEPR1/2* promoter-mediated GUS expression was observed in root tips. Focusing on the expression in roots, the activity of the *PEPR2* promoter was more restricted to the central cylinder of the root, whereas GUS expression of the *PEPR1* promoter was present in most root tissues. Additionally, GUS expression mediated by the *PEPR1/2* promoters was detected in stems but was almost absent in flowers.

When comparing the expression patterns between the receptors and the precursors, the *PEPR1/2* promoter-mediated expression showed partially overlapping patterns with *PROPEP1*, *-2*, *-3*, *-5*, and *-8*. By contrast, *PROPEP4* and *-7* promoter-mediated expression was exclusive to the root tip, a tissue where the *PEPR*s were not expressed. These results showed that, whereas the promoters of *PEPR1* and *-2* significantly overlapped in their conferred expression patterns, they shared only a small overlap with the expression patterns generated by the *PROPEP* promoters. This indicates potential new, unknown roles for at least PROPEP4 and -7 independent of PEPRs.

PEPR1 and -2, as well as all eight At*Peps, trigger similar defence responses*

Previous studies showing that *At*Peps triggered alkalinization in cell cultures and induced resistance to *P. syringae* infection in plants provided evidence that some *At*Peps are functionally redundant (Huffaker and Ryan, 2007; Yamaguchi *et al.*, 2010). To address the extent of functional redundancy among all known *At*Peps, we monitored the activation of MAPK, the release of ethylene, and the inhibition of seedling growth stimulated by the eight *At*Peps in the single and double *pepr1 pepr2* receptor mutants. As shown in Fig. 6, all eight *At*Peps activated the stress-related MAPKs MPK3 and -6, induced the production of ethylene, and inhibited seedling growth in a PEPR1- and partially PEPR2-dependent manner. Notably,

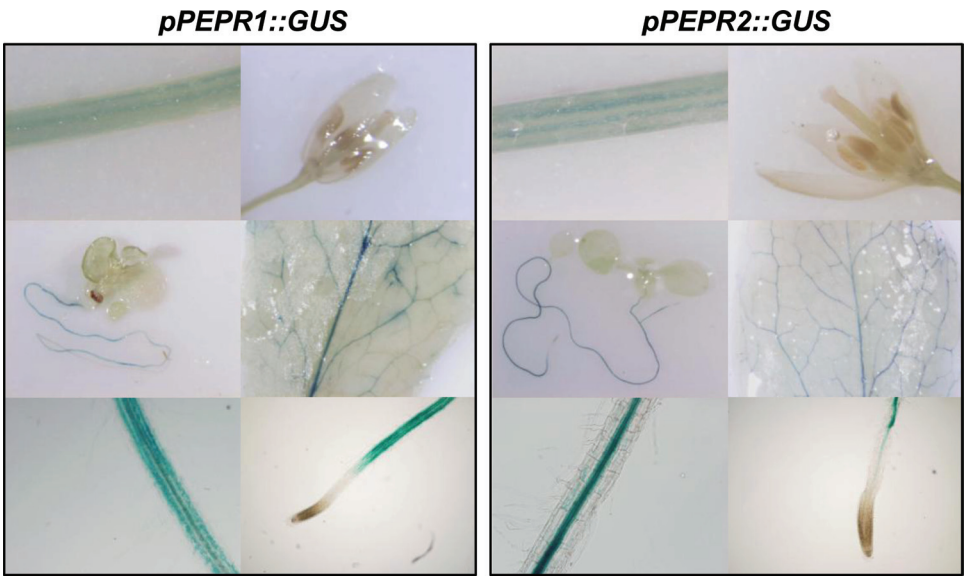

Fig. 5. Overlapping expression patterns of *PEPR1* and *-2* promoter–GUS lines. Putative promoter sequences of *PEPR1* and *-2* were fused to GUS and stably introduced into *Arabidopsis* plants. Tissues of transgenic plants were stained for 2 h (roots and seedlings) or 24 h (adult leaves, stems, and flowers). GUS staining revealed a significant overlap in the tissue-dependent expression of *PEPR1* and *-2*. Three independent transgenic lines were analysed for each construct. Pictures show representative samples.

AtPep3–8 were not perceived in the *pepr1* mutant, indicating that PEPR2, which is active in this mutant, does not perceive these peptides and thus is specific for *At*Pep1 and -2, whereas the *pepr2* mutant responded to all peptides, indicating that PEPR1 recognizes all eight *At*Peps in a similar way (Fig. 6). Taken together, all eight *At*Peps triggered a similar set of defence responses reminiscent of PTI in a PEPR1- and partially PEPR2-dependent manner. Thus, in contrast to the PROPEPs, the *At*Peps as well as PEPRs appear to be highly redundant.

Discussion

Current models discuss PROPEPs and *At*Peps as: (i) enhancers of immunity; (ii) damage-signalling peptides; and (iii) elicitors of systemic defence responses, but based on published data, reliable support for each model is scarce (Boller and Felix, 2009; Yamaguchi and Huffaker, 2011). Previous studies focused primarily on plant responses triggered by the addition of the synthetically produced peptides AtPep1 or -3 and firmly established that treatment with these peptides enhances plant immunity via PEPRs (Krol *et al.*, 2010; Yamaguchi *et al.*, 2010). Likewise, the constitutive, ubiquitous expression of *PROPEP1* or *-2* improved plant resistance to an oomycete pathogen (Huffaker *et al.*, 2006). However, these studies did not address the question of the presence or the underlying mechanism of the PROPEP/*At*Pep/PEPR system and thus cannot fully answer which (if any) of the current models is valid.

Recently, two studies involving the *pepr1 pepr2* double mutant suggested an interaction of *At*Pep signalling with the defence hormone ethylene to maintain PTI responses (Liu *et al.*, 2013; Tintor *et al.*, 2013). Thus, the 'enhancer of immunity' model appears now to be the most likely one. Here, we investigated the presence and regulation of PROPEPs to either further substantiate the 'enhancer model' or to deduce new biological role(s) of the PROPEP/*At*Pep/PEPR system.

PROPEP1, -2, -3, *and maybe* -5 *and* -8 *play a role in immunity*

In agreement with previous works (Huffaker and Ryan, 2007; Yamaguchi *et al.*, 2010), our bi-clustering showed that *PROPEP1*, *-2*, and *-3* clustered together with genes implicated in plant defence. Moreover, the almost exclusive expression of these *PROPEP*s in the roots revealed by promoter::GUS fusions partially overlapped with those of *PEPR1* and *-2*. Thus, *PROPEP1*, *-2*, and *-3* might play specific roles in the immune response of the root, which is supported by the report that constitutive expression of *PROPEP1* led to an induced resistance against the oomycete root pathogen *Pythium irregulare* (Huffaker and Ryan, 2007). In contrast, these *PROPEP*s are not or are only weakly expressed in adult leaves but are rapidly induced in wounded leaf veins. Recently, we showed that pre-treatment of leaf tissue with bacterial MAMPs led to enhanced output of reactive oxygen species triggered by *At*Pep perception (Flury *et al.*, 2013). Since a progressive wave of reactive oxygen species has been discussed as a potential systemic signal, the enhanced expression of PROPEPs in wounded vasculature might contribute to the robustness of this system (Miller *et al.*, 2009; Mittler *et al.*, 2011).

PROPEP5 and *-8* displayed expression patterns that partially overlapped with those of *PEPR1* and *-2*, but, in contrast to *PROPEP1*, *-2*, and *-3*, *PROPEP5* and *-8* were restricted to the root vasculature but were more expressed in the leaf veins (*PROPEP5*) and the flowers (*PROPEP8*). However, *PROPEP1*, *-2*, *-3*, *-5*, and *-8* together cover most plant tissues and, since all eight *At*Peps triggered redundant responses, PROPEP5 and -8 could play a role in leaves and flowers, respectively, similar to the roles of PROPEP1, -2, and -3 in roots.

It has been hypothesized that PROPEPs are located to the cytoplasm and could be released into the extracellular space in a situation of danger using unconventional protein secretion mechanisms (Ding *et al.*, 2012). We indeed found that PROPEP3::YFP was localized in the cytoplasm, but surprisingly PROPEP1::YFP as well as PROPEP6::YFP were detected at the tonoplast. Due to the acidic environment of the vacuole negatively impacting on YFP fluorescence, we assume that PROPEP1::YFP and PROPEP6::YFP are associated with the cytoplasmic side of the vacuolar membrane. Notably, the localization signal that directs the PROPEP to the tonoplast or to a hitherto unidentified interaction domain that could attach the PROPEP to a tonoplast-localized protein, resides in the N terminus of the PROPEP, since a fusion protein of only AtPep1 and YFP localized in the cytoplasm. Therefore, it can be excluded that the *At*Pep itself binds to a tonoplast-localized receptor-like protein. Recently, it was shown that infection of *Arabidopsis* with the compatible oomycete *Hyaloperonospora arabidopsidis*, the causal agent of the downy mildew, triggered rearrangement of intracellular membranes leading to relocation of the tonoplast close to the extra-haustorial membrane (Caillaud *et al.*, 2012). However, neither the involvement of PROPEPs in resistance to *H. arabidopsidis* nor the necessity of a tonoplast localization of PROPEP1 in the context of resistance to *Pythium* infection has yet been shown, but it will be interesting to study this potential connection.

Root tip-expressed PROPEP4 *and -7 are distinct from the other PROPEPs and may have dual functions*

PROPEP4 and -7 are located on chromosome 5 within an ~3.5 kb stretch. Both share specific expression in the tips of primary and lateral roots, which does not overlap with that of *PEPR1* and *-2*. Moreover, they are currently the only *PROPEP*s that are not induced by wounding. Therefore, they are less likely to enhance plant immune responses locally. However, PROPEP4 and -7 could still be part of a systemic defence response. It has been reported that the systemin peptide is transported via phloem sap (Narváez-Vásquez *et al.*, 1995). Moreover, a plethora of peptide transporters are encoded in the *Arabidopsis* genome and might facilitate the transport of *At*Peps for systemic signalling (Stacey

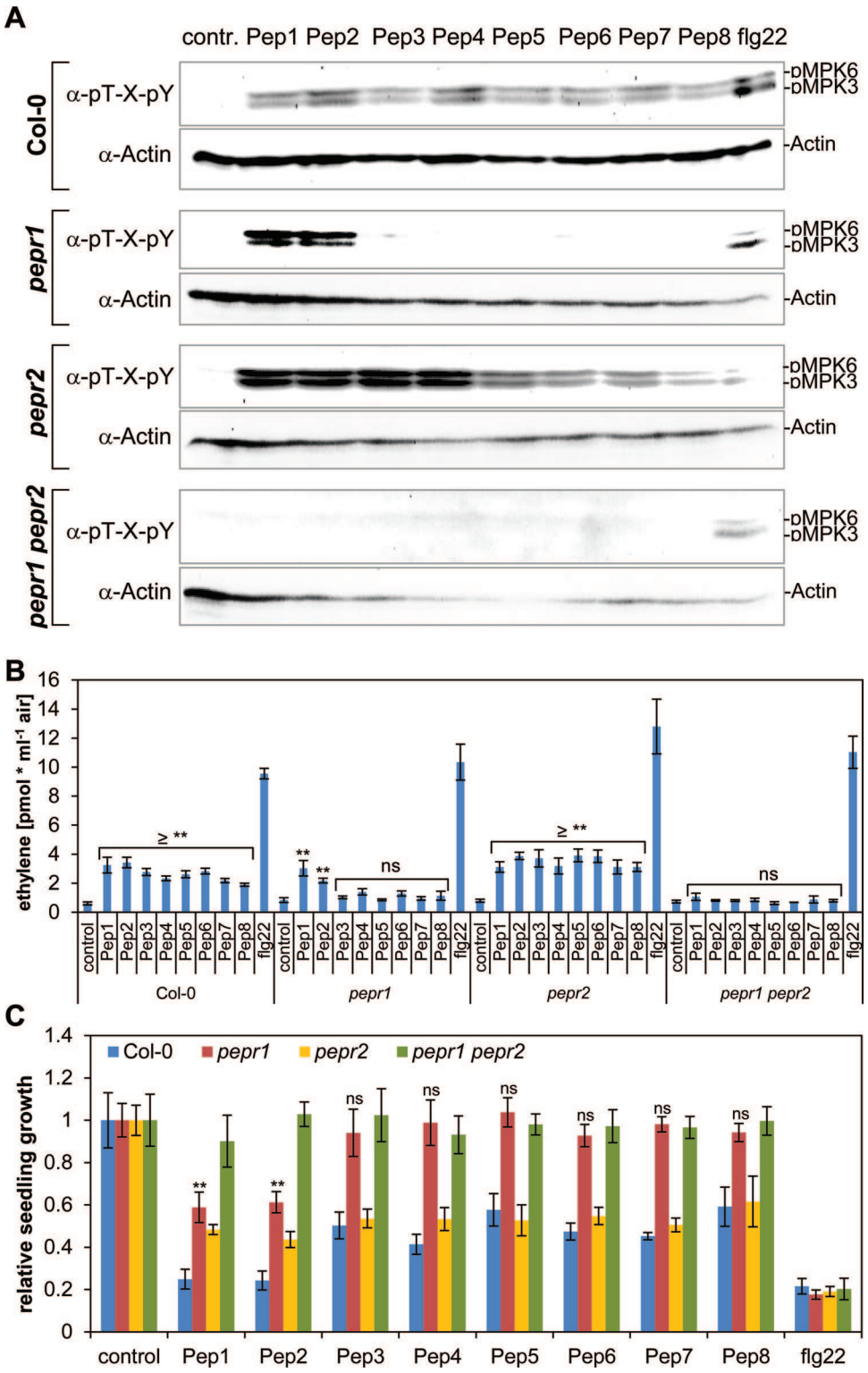

Fig. 6. Defence responses activated by all eight *At*Peps and both PEPRs. (A) MAPK phosphorylation. Seedlings of the indicated genotypes were treated for 15 min with 1 μM of the indicated elicitor peptide or without any peptide (contr.). MAPK phosphorylation was detected by immunoblotting using an anti-phospho-p44/42-MAPK antibody detecting the pTE-pY motif of MPK6 and -3. The immunoblot was reprobed with anti-actin antibody to determine equal loading. (B) Ethylene production. Seedlings of the indicated genotypes were treated for 5 h with 1 μM of the indicated elicitor peptides or without any peptide (control). Columns represent averages of detected ethylene values of five biological replicates. Error bars indicate SEM.(C) Seedling growth inhibition. Five-d-old seedlings of the indicated genotypes were treated for 5 d with 1 μM of the indicated elicitor or without any peptide. Columns represent the mean weight of 12 seedlings out of six biological replicates. Error bars indicate SEM. Asterisks represent *t*-test results generated by comparing the labelled value with the respective control $^{**}P<0.01$; ns, not significant). (This figure is available in colour at *JXB* online.)

et al., 2002). Thus, PROPEP4 and -7 could be ideal candidates to study whether PROPEPs or *At*Peps are transported systemically.

The Affymetrix 25K microarrays do not represent *PROPEP7*. Our bi-clustering analysis produced only a small cluster of 25 genes that contained *PROPEP4*. Intriguingly, this cluster showed an expression pattern opposite to those of the other *PROPEP*-containing clusters, meaning that whenever biotic stress treatments lead to an induction of *PROPEP4* expression, other *PROPEP*s are downregulated and vice versa. GO term enrichment indicates biological processes including chromatin and chromosome organization. However, this does not exclude a function in immunity. The mammalian DAMP high-mobility group protein B1 (HMGB1) binds to DNA, modifies the shape, and regulates transcription. In case of danger, it can be secreted by activated monocytes and macrophages, or it is passively released by necrotic or damaged cells. Detection of extracellular HMGB1 by RAGE (receptor for advanced glycation end products) of adjacent cells triggers inflammation (Scaffidi *et al.*, 2002; Sims *et al.*, 2010).

The small *PROPEP4*-including gene cluster also shows the limitations of the bi-clustering. Most of the arrays used were probed with samples based on seedlings or adult leaves. Genes with tissue-restricted expression patterns like *PROPEP4* might only be detected weakly on some of the biotic stress arrays, leading to erroneous expression patterns.

Taken together, PROPEP4 (and PROPEP7) are clearly distinct from the other PROPEPs in terms of tissue expression pattern as well as regulation within the biotic stress treatments. A more detailed analysis is needed to uncover their biological roles.

PROPEPs may play roles in plant reproduction and development

Most plant signalling peptides originate from small (~100 aa) proteins, which are processed at the C terminus to release the active signalling peptide. These peptides have various functions, especially in developmental processes such as apical meristem development as well as root growth (Matsubayashi and Sakagami, 2006; Katsir *et al.*, 2011). PROPEPs have been associated with plant innate immunity but share structural similarities (size and presence of signalling peptide in the C terminus) with *Arabidopsis* signalling peptide precursors like RGF1, TDIF, CLV3, PSK1, CEP1, and PSY1. Remarkably, there may be also a functional overlap. In contrast to *PROPEP1*, *-2*, and *-3*, bi-clustering showed that *PROPEP5* clusters with genes associated with plant reproduction. Although we did not find this for *PROPEP3*, GUS analysis revealed expression of both in the stamen. Thus, beside the proposed role in plant immunity, *PROPEP5* and maybe also *PROPEP3* could be involved in the development of the stamen and therewith in the regulation of reproduction. The involvement of small signalling peptides in this process has been demonstrated just recently. RALF (rapid alkalinization factor) signalling peptides regulate pollen-tube elongation and development of the female gametophyte in *Solanaceous* species (Covey *et al.*, 2010; Chevalier *et al.*, 2013). Thus, as a next step, a detailed analysis of *PROPEP3* and *-5* knockout mutants would be needed to investigate a potential role of these *PROPEP*s in plant reproduction.

Besides the impact of constitutive expression of *PROPEP1* on resistance against *Pythium* infection, it also promoted an increase in root biomass production (Huffaker *et al.*, 2006). It was assumed that *PROPEP1* expression somehow generated an advantage for *Arabidopsis* roots to grow in soil. In contrast, exogenous application of *At*Peps blocked root growth and biomass production similarly to seedling growth inhibition triggered by MAMPs. Notably, application of *At*Peps has a more pronounced negative effect on root growth compared with MAMPs (Krol *et al.*, 2010).

Whether root growth is also enhanced in sterile conditions by constitutive expression of *PROPEP1* has been not assessed (Huffaker *et al.*, 2006); thus, this advantage may or may not be based on an increased pathogen resistance of the root. A detailed analysis of *propep1* knockout mutants is needed to clarify whether PROPEP1 takes part in additional processes like root development.

Conclusions

Previous studies and our new data reported here show that all eight *At*Peps trigger a PTI-like response by binding to either PEPR1 or both PEPR1/2 receptors (Huffaker and Ryan, 2007; Yamaguchi *et al.*, 2010). Interestingly, PEPR2 is specific for AtPep1 and AtPep2, whereas PEPR1 is non-specific and recognizes all eight *At*Peps.

In contrast to the *At*Peps and the PEPRs, we have provided data indicating that PROPEPs are probably not redundant. They show individual spatial and temporal expression patterns and localize to distinct subcellular compartments. Besides their potentially diverse roles in innate immunity, they may additionally be involved in plant development and reproduction. A detailed characterization of each PROPEP, together with an analysis of their processing and release, will be necessary to uncover the full array of functions of PROPEPs in plant biology.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table 1. GO term enrichment for selected *PROPEP*-containing clusters in Fig. 2.

Supplementary Table 2. GO term enrichment for genes co-expressed with *PROPEP*s under biotic, abiotic or developmental conditions.

Supplementary Table 3. Conditions and/or treatments influencing the expression of each *PROPEP*.

Supplementary Table 4. Primer and promoter sequences.

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